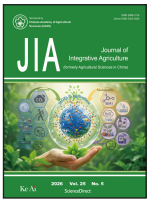
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LETTER

Generating popcorn-like fragrant tomato using CRISPR/Cas9-mediated gene editing

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Highlights

- CRISPR/Cas9-mediated simultaneous knockout of both *SlBADH1* and *SlBADH2* led to the development the first extraordinary aromatic tomato plants.
- None of the edited lines differed significantly from the wild type (WT) for any key agronomic or quality trait.

Tomato (*Solanum lycopersicum* L.) is one of the world's most widely cultivated and consumed vegetables, and its fruits are highly valued for its vibrant color, culinary versatility, and nutritional benefits (Yu *et al.* 2025). China leads global tomato production, with an output of approximately 70 million tons in 2023, contributing to a worldwide yield exceeding 192 million tons (FAO 2025). Fragrance significantly enhances the sensory appeal and flavor quality of food, thereby influencing the consumption and market price of agricultural products. For example, fragrant rice (*Oryza sativa* L.) varieties like Basmati and Jasmine have gained increasing popularity worldwide due to their distinct aroma, and their prices are often more than twice that of regular rice (Shan *et al.* 2015). Among the more than 200 volatile organic compounds (VOCs) characterized in aromatic rice, 2-acetyl-1-pyrroline (2-AP) is a dominant VOC responsible for the delightful "popcorn-like" aroma (Okpala *et al.* 2019). This fragrance is produced by loss-of-function mutations in the *betaine aldehyde dehydrogenase 2* (*BADH2*) gene (Imran *et al.* 2023). In non-fragrant rice, *BADH2* converts γ-aminobutyraldehyde (GABald) to γ-aminobutyric acid (GABA), while in fragrant varieties, the disrupted activity of *BADH2* leads to accumulation of GABald, which is subsequently converted to 2-AP (Hui *et al.* 2022).

CRISPR/Cas9 genome editing has recently facilitated the development of fragrant varieties in multiple crops by targeting *BADH2* homologs, including monocots: *Zea mays* (Wang *et al.* 2021), *Sorghum bicolor* (Zhang *et al.* 2022), and *Setaria italica* (Zhang *et al.* 2023), and eudicots: *Glycine max* (Xie *et al.* 2024), and *Arachis hypogaea* (Xue *et al.* 2025). However, no naturally popcorn-like fragrant tomato genotype has yet been discovered, and centuries of domestication and intensive breeding have systematically eroded flavor in commercial cultivars (Zhang *et al.* 2024; Wu *et al.* 2025). Given the global significance of tomato as a popular fruit source, we aimed to investigate whether the function of *BADH2* is conserved in tomato and whether fragrant tomato germplasm can be produced through targeted disruption of tomato *BADH2* homolog.

To identify *BADH2* homologs in tomato, we screened the tomato reference genome (https://solgenomics.net/organism/Solanum_lycopersicum/genome/) for proteins exhibiting high similarity to both OsBADH1 and OsBADH2. Through BLAST analysis, two putative *SlBADH* genes were identified in tomato, namely *Solyc03g113800* and *Solyc06g071290*. Phylogenetic analysis of BADH1 and BADH2 proteins from diverse plant species classified them into four clades (Appendix A). Monocot and eudicot BADHs clustered separately in clades A and C, respectively. Clade B contained basal angiosperms and gymnosperms, while clade D included gymnosperms, lycophytes, mosses, and algae, suggesting functional divergence among these proteins.

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Within clade C, neither Solyc03g113800 nor Solyc06g071290 showed a closer phylogenetic relationship with known BADH2 proteins (Appendix A). Therefore, we demonstrated the homology of tomato BADH2 based on sequence identity. Notably, Solyc06g071290 showed higher sequence identity with *BADH1* genes from rice, foxtail millet, Arabidopsis, and soybean, with sequence identities of 66.98, 67.35, 68.09, and 68.83%, respectively (Appendix B-a). In contrast, Solyc03g113800 displayed greater similarity to BADH2. Consequently, *Solyc06g071290* and *Solyc03g113800* were defined as *SlBADH1* and *SlBADH2*, respectively. We then examined the expression pattern of both *SlBADH1* and *SlBADH2* gene using RNA-seq data from MicroTom accession. Interestingly, *SlBADH1* exhibited lower and tissue-specific expression, while *SlBADH2* was expressed throughout all growth stages and all tissues examined. The expression of *SlBADH2* was particularly high in fruits at early developmental stages, while relatively low in leaves and fruits at later stages (Appendix B-b).

Given that *BADH1* has also been involved in fragrance generation (Amarawathi *et al.* 2008; Xue *et al.* 2025), we investigated the function of both *SlBADH1* and *SlBADH2* in tomato. The CRISPR/Cas9-mediated genome editing technology was used to knock out individuals or both *SlBADH1* and *SlBADH2* gene in the variety AC (Alisa Craig). Detailed materials and methods are provided in Appendix C. We designed two specific target sites in the first exon and second exon of *SlBADH1* and *SlBADH2*, respectively (Fig. 1-A and B). The two sgRNAs with target sequences were individually driven by AtU6-1 and AtU6-29 promoters and cloned into the CRISPR/Cas9 vector pYLCRISPR/Cas9Pubi-B (Fig. 1-C). The CRISPR/Cas9 expression vector was transformed into tomato AC through *Agrobacterium tumefaciens*-mediated stable transformation, resulting in 20 independently positive transformants in the T_0 generation. The target genomic regions were PCR-amplified, and followed by Sanger sequencing. Ultimately, we identified 11 T_0 plants (55%) with mutations in either or both of the target genes (Appendices D and E).

Cas9-free *slbadh* mutant lines were selected in the T_1 generation. Sequencing results revealed that the *slbadh1-1* and *slbadh1-2* lines had 4-bp deletion and 1-bp deletion in *SlBADH1*, respectively (Fig. 1-D), while the *slbadh2-1* and *slbadh2-2* lines harbored 1-bp insertion and 5-bp deletion in *SlBADH2*, respectively (Fig. 1-E). The double mutant line *slbadh1/2-1* had a 5-bp deletion in both genes, while the *slbadh1/2-2* contained a 1-bp insertion in both *SlBADH1* and *SlBADH2* (Fig. 1-F). All mutations caused frameshifts, expected to impair function of *SlBADHs*. To assess off-target activity, we Sanger-sequenced the three highest-ranking CRISPR-P 2.0-predicted off-target loci (Liu *et al.* 2017b) for each sgRNA in the representative mutant lines. No off-target events were detected at any of these sites (Appendix F).

The tomato plants were cultivated in a greenhouse under the photoperiod of 14 h/10 h (light/dark), with temperatures at 25°C/20°C (light/dark). None of the six single or double mutant lines differed significantly from the wild type (WT) for any key agronomic or quality trait including flowering time, plant height, fruit weight, soluble sugars (glucose, fructose, sucrose), organic acids (citric and malic acid), or vitamin C content (Fig. 1-G and H; Appendices G and H-d). Sensory evaluation by a panel of 10 volunteers revealed that fruits and leaves of *slbadh2* and *slbadh1/2* mutant lines had a distinct popcorn-like aroma, while fruits and leaves of WT and *slbadh1* mutant lines lacked this fragrance. We further quantified the 2-AP content in tomato plants using liquid chromatography-mass spectrometry (LC-MS). The results showed that the *slbadh2* but not the *slbadh1* single mutant lines exhibited a significantly higher 2-AP content. Notably, 2-AP content in both leaves and fruits of the double mutants was over four times higher than those in the *slbadh2* single mutants (Fig. 1-I and J). These results indicated that although *SlBADH2* played a predominant role in regulating the 2-AP accumulation in tomato, *SlBADH1* also significantly contributed to this regulatory process. Similar conclusions were also drawn in the studies on *GmBADHs* (Xie *et al.* 2024) and *AhBADHs* (Xue *et al.* 2025). A reasonable explanation for the significant elevation of 2-AP content in the *slbadh1/2* double mutants is that SlBADH2 is the primary enzyme responsible for GABald conversion in tomato, while SlBADH1 may have lower expression or catalytic efficiency toward this substrate. In the double mutants, the complete disruption of both genes leads to maximal GABald accumulation and thus the highest 2-AP production.

In Arabidopsis, *aldh10a9* (*BADH* gene) mutants are more sensitive to salt stress than WT (Zarei *et al.* 2016). However, the results of NaCl treatment with different concentrations in tomato suggested that the single or double mutations of *SlBADHs* may not exhibit higher sensitivity (Appendix H), consistent with earlier observations in *Brassica napus* (Wu *et al.* 2024).

To investigate whether natural variation in the *SlBADH2* gene contributes to fragrant germplasm resources, we conducted haplotype analysis by screening the *SlBADH2* sequences of 706 tomato accessions (Zhou *et al.* 2022), and focused on variations in the exons. A total of 13 single nucleotide polymorphisms (SNPs) were found in the exon of *SlBADH2*, of which 4 resulted in single amino acid substitutions. Based on these 4 variations, all tomato accessions were classified into 4 haplotypes (Appendix I). We randomly selected 2 accessions from each haplotype to perform fragrance sensory test using leaves, and found that none of them were fragrant (Fig. 1-K; Appendix I). Therefore, our results suggested that the natural mutations of *SlBADH2* did not affect gene function and naturally popcorn-like aromatic tomato seem to be absent.

In summary, our findings demonstrate that *SlBADH2* is a dominant gene in controlling 2-AP biosynthesis in tomato, and *SlBADH1* plays a positive role in the accumulation of this compound. We generated the first extraordinary aromatic tomato plants by simultaneously mutating both *SlBADH1* and *SlBADH2* genes using CRISPR/Cas9. Ongoing work aims to introduce this fragrance to elite commercial cultivars, which may enhance their flavor complexity, potentially improving consumer preference and market value, like fragrant rice varieties.

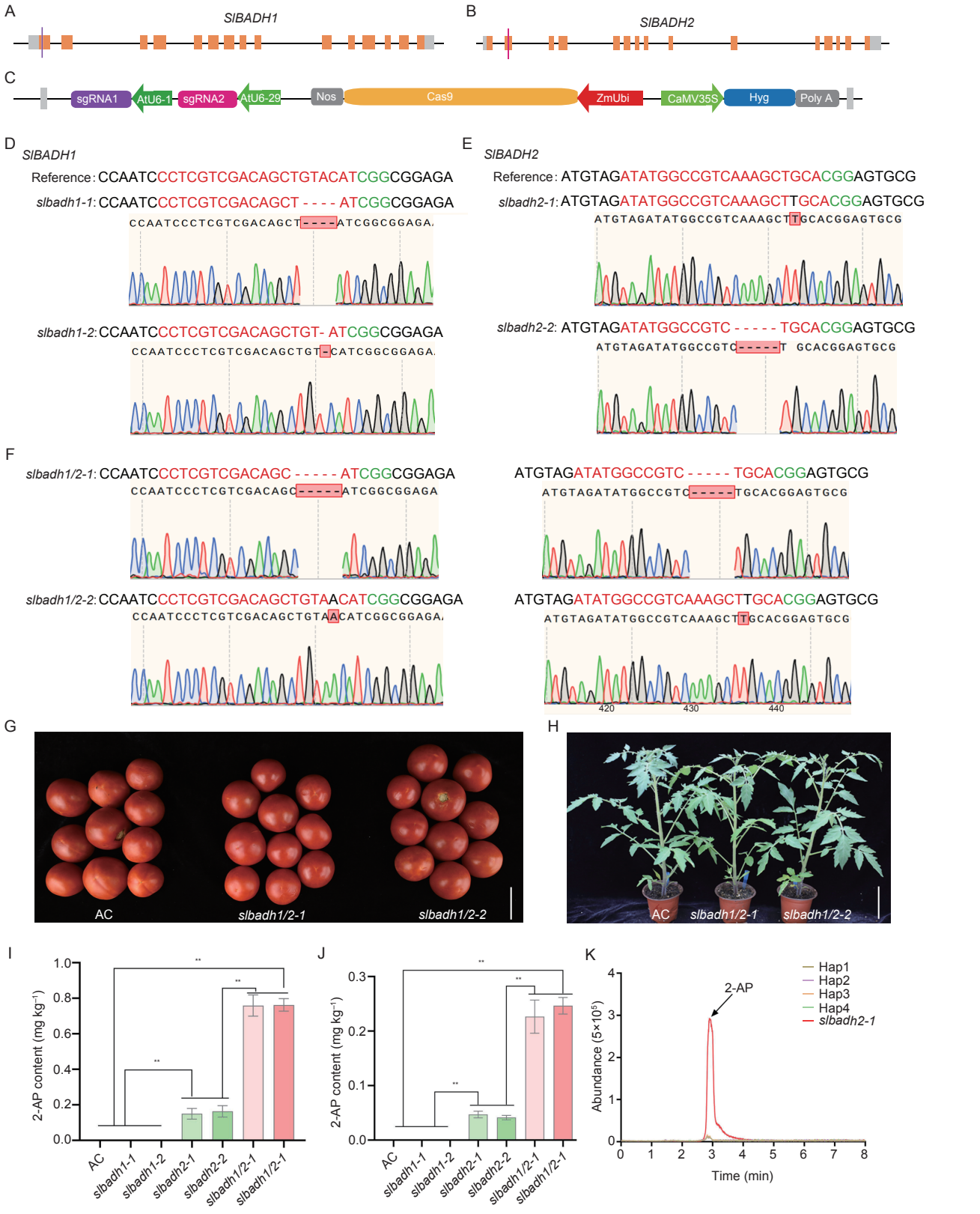


Fig. 1 Creation of fragrant tomato with elevated 2-AP content through CRISPR/Cas9-mediated disruption of *SlBADH1* and *SlBADH2*. A, gene structure and the target site design (purple line) of *SlBADH1*. B, gene structure and the target site design (red line) of *SlBADH2*. C, schematic diagram of the T-DNA region of the CRISPR/Cas9 vector. Hyg, hygromycin phosphotransferase. D–F, mutation analysis of *slbadh1* (D), *slbadh2* (E), and *slbadh1/2* (F). The protospacer adjacent motif sequence and target sites are indicated in green and red, respectively. G, photograph of mature fruits of AC (wild-type), *slbadh1/2-1* and *slbadh1/2-2*. Scale bar=3 cm. H, photograph of AC, *slbadh1/2-1* and *slbadh1/2-2* at 30 days after emergence (DAE). Scale bar=10 cm. I and J, the 2-AP content in fresh leaves of 1-month-old plants (I) and fruits at Br+12 stage (J) of AC and the mutants. The data contain three independent biological replicates from different plants; the error bars represent the SD. *P*-values were calculated using ANOVA. **, $P<0.01$. K, total ion chromatograms of 2-AP in leaves of the accession from each haplotype of *SlBADH2*. The details of each haplotype are shown in Appendix I.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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